

RESEARCH ARTICLE



Impact of prematurity on LINE-1 promoter methylation

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ABSTRACT

Aim: Promoter methylation of LINE-1 may be affected by prematurity, but there is little evidence in the literature.

Materials & methods: Blood from premature and full-term neonates on days 0, 5, 30 and 90 was analyzed for DNA methylation percentage in a promoter region of the LINE-1, after bisulfite conversion and pyrosequencing.

Results: Premature infants, as a whole, showed significantly lower methylation percentage at birth, but this difference diminished over time. However, the subgroup of extremely premature (<28 weeks gestational age) had higher methylation percentages, similar to full-term newborns.

Conclusion: This research underscores the critical role of prematurity on the methylation pattern of LINE-1. These findings underline the complexity of epigenetic regulation in prematurity and emphasize the need for further studies.

PLAIN LANGUAGE SUMMARY

Premature birth can have significant effects on a baby's development and long-term health. This study investigates how being born prematurely affects a process called DNA methylation, which can influence how genes are turned on or off. Specifically, we examined the LINE-1 promoter, a frequently occurring region of DNA known for its role in regulating gene activity.

We collected blood samples from both premature and full-term newborns at birth and at several points in the early months of life. Our findings showed that premature babies have lower levels of LINE-1 promoter methylation at birth compared with full-term babies. These differences in methylation could possibly affect the babies' development and health as they grow.

Our research highlights the need for continued study in this area to explore how these epigenetic changes impact long-term health and to develop strategies to mitigate these effects.

TWEETABLE ABSTRACT

Premature neonates show lower LINE-1 methylation at birth, which increases to full-term newborn levels over the first days of life. Our study highlights the impact of prematurity on initial epigenetic configuration. #Prematurity #Epigenetics #DNAmethylation

ARTICLE HISTORY

Received 16 June 2024
Accepted 20 August 2024

KEYWORDS

DNA methylation;
epigenetics; LINE-1;
newborn; prematurity;
retroelements

1. Introduction

Globally, one in every ten neonates is born prematurely (before 37 weeks of gestation) [1]. This situation is alarming, with about 1 million premature infants dying annually due to complications from early birth, making prematurity the leading cause of death among children under 5 years old in the world [2]. Prematurity is

categorized into three levels, depending on gestational age: extremely premature (<28 weeks of gestation), very premature (≥ 28 to <32 weeks of gestation) and moderately premature (≥ 32 to <37 weeks of gestation) [3,4].

Recent research has explored the connection between prematurity and epigenetics, highlighting how epigenetic modifications can influence susceptibility to prematurity and associated health outcomes [5,6]. The

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growing interest in epigenetic variations has led to significant discoveries about their association with a wide range of diseases, including autoimmunity, cancer, intellectual disabilities, endocrine and neuropsychiatric disorders [5,7,8]. These findings highlight the critical role of epigenetics in understanding the molecular basis of various health conditions [9]. Concurrently, transposable elements (TEs), DNA sequences capable of moving within the genome, have been the subject of intense research. Long Interspersed Nuclear Elements (LINE-1) make up about 18% of the genomic DNA and are autonomous retrotransposons [10,11], and consist of a promoter sequence, two ORFs and a poly-A tail [12,13]. This element plays a vital role in generating genetic material mosaicism in neuronal cells and is associated with genomic instability and brain development [14]. Although most LINE-1 elements in the human genome are inactive, a small number are still mobile and can cause significant genomic variations [12,14,15].

During embryonic development, active LINE-1 elements can be expressed in stem cells and influence gene regulation and cellular diversity, playing a significant role in embryogenesis [16]. They contribute to genetic diversification and adapt to the specific demands of different developmental stages [17]. However, excessive or deregulated activity of LINE-1 can be harmful, leading to mutations, disruptions in gene expression and cellular dysfunction, resulting in abnormalities in embryonic development and congenital disorders [14,18,19]. The LINE-1 promoter is often used to assess the global methylation status of the genome [20,21]. Changes in the methylation of these repetitive elements can influence genomic stability and gene expression, offering valuable insights into epigenetic alterations that affect the genome in different contexts, such as embryonic development, aging and diseases [22,23]. Intragenic LINE-1 methylation plays an essential role in controlling gene expression during development [16,17]. Although LINE-1 elements are abundant in the genome, the majority are 5' truncated and do not contain a functional promoter region [24,25]. This study aims to specifically measure the methylation in the promoter region of LINE-1, which actually represents only a fraction of the LINE-1 loci in the genome.

Prematurity has been linked to various adverse health outcomes later in life, including respiratory problems, neurodevelopmental disorders and metabolic diseases [26]. Epigenetic modifications, such as changes in DNA methylation, play a significant role in mediating these long-term effects [5]. Investigating the methylation profile of the LINE-1 element in premature infants is important for understanding the impact of epigenetic modifications on neonatal health and development.

Analyzing LINE-1 methylation aids in comprehending how exposure to adverse conditions in premature birth influences infant epigenetics, potentially leading to unfavorable health outcomes [11,27]. Factors such as maternal stress, inflammation and inadequate nutrition during pregnancy can affect fetal DNA methylation, with LINE-1, a common repetitive element in the genome, being particularly sensitive to these epigenetic changes [28]. Maternal nutrient deficiencies, particularly in methyl donors such as folic acid, have been shown to significantly influence the epigenetic landscape of the developing fetus [29]. Adequate levels of folic acid during pregnancy are crucial for proper DNA methylation processes, including those involving LINE-1 elements [30]. Deficiency in these nutrients can lead to hypomethylation, which is associated with various adverse developmental outcomes, including increased susceptibility to prematurity and its related complications [31,32].

The primary aim of this study is to describe the methylation percentage pattern of the LINE-1 promoter in full-term and preterm neonates at different time points following birth.

2. Materials & methods

2.1. Study design, study population & ethics

This is an observational, longitudinal and prospective study involving 46 preterm (less than 32 weeks gestational age, $\leq 1,500$ g and appropriate for gestational age) and 49 full-term newborns from a cohort born between April 2018 and May 2019, previously described [33]. The group of premature infants was subdivided into two subgroups, according to the World Health Organization classification (<https://www.who.int/news-room/fact-sheets/detail/preterm-birth>): (1) very preterm (28 to less than 32 weeks gestational age) and (2) extremely preterm (less than 28 weeks). Gestational age was primarily determined by first-trimester ultrasound or, in cases where this was unavailable, by the estimate provided by the last menstrual period and by the Capurro or Ballard clinical assessment methods [34,35]. Newborns with major congenital malformations, clinical suspicion or laboratory confirmation of genetic syndromes were excluded. Data from patients who died were considered up to the point of their participation. The study was approved by the Research Ethics Committees of the institutions involved, and all parents or legal guardians consented to their children's participation through the signature of written informed consent. All data have been anonymized.

2.2. Biospecimen collection

Blood samples, totaling 1 to 2 ml from the umbilical cord at birth or 0.5 ml from peripheral veins on the fifth day post-birth, at 1 month of age and upon hospital discharge, were collected in tubes containing potassium EDTA as an anticoagulant. After collection, the material was stored in refrigerators at 4–8°C for up to 48 hours. Then, the tubes were transported in thermal boxes, maintaining a temperature range of 2–8°C, to the epigenetic laboratory, where blood from each tube was divided into 0.5 ml aliquots, promptly identified and stored in an ultra-freezer at -80°C until further processing stages.

2.3. Global (LINE-1) DNA methylation analysis

The DNA was extracted from blood mononuclear cells employing the *DNeasy Blood & Tissue Kit* (Qiagen, Hilden, Germany). Subsequently, the quantity and purity of the DNA were assessed using a *NanoDrop™ spectrophotometer 2000c* (ThermoFisher Scientific, MA, USA). Following this, 1000 ng of DNA underwent bisulfite conversion utilizing the *EZ-96 DNA Methylation Kit* (Zymo Research, CA, USA). Per the manufacturer's protocol, the converted DNA was eluted with 24 μ l of the M-elution buffer. Next, 2 μ l of bisulfite-converted DNA was amplified via PCR using the *PyroMark PCR kit* (Qiagen) in a total volume of 50 μ l, including 0.2 μ M of primers and PyroMark PCR Master Mix. Confirmation of DNA amplification was achieved through 3% agarose gel electrophoresis. Biotinylated PCR products in a total volume of 10 μ l were immobilized on 3 μ l of magnetic streptavidin-coated Sepharose beads (PyroMark Q48 Magnetic Beads). Subsequently, pyrosequencing was performed using PyroMark Q48 Advanced CpG reagents on the PyroMark Q48 Autoprep system (both from Qiagen). The methylation percentage of each CpG site was automatically generated utilizing the PyroMark Q48 Autoprep Software (v. 2.4.2) with standard quality control settings. The design of PCR and pyrosequencing primers was carried out using PyroMark Assay Design 2.0 (Qiagen).

2.4. Selection of the target region

The four CpG sites examined (CpGs 21, 22, 23 and 24) were investigated using the revised genomic consensus for the CpG island region in the LINE-1 promoter in humans (L1-HS) [36]. The primers design was performed to ensure that the amplified region would be compatible with the parameters of the PyroMark Q48 Autoprep from Qiagen (Hilden, Germany), spanning from nucleotide 222 to 360, corresponding to the accession code X58075.1 in the NCBI GenBank (Table 1).

2.5. Statistical analysis

The data related to the studied samples were cataloged and organized in .csv format, and all statistical procedures were conducted using the Python 3.7 programming language (Python Software Foundation, Wilmington, Delaware, USA) in a Jupyter Notebook environment. The primary libraries utilized included Pandas, Numpy, Scipy and Seaborn. In the descriptive analysis of the epidemiological and clinical characteristics of the pregnant women and the term and preterm newborns, the Mann-Whitney U test was applied for numerical variables and the chi-square test or Fisher's exact test for categorical variables. For the descriptive analysis of methylation percentage, continuous variables were presented as median and interquartile ranges of 25% and 75% or mean and standard deviation, depending on the data distribution. Median comparisons between groups were conducted using the Mann-Whitney test. The results of global methylation were visualized in box plot and violin plot graphs. The temporal evolution of methylation at each CpG site was determined by the average methylation for each group, with the data plotted in point graphs and a trend line obtained through linear regression, including R^2 , slope and p -value estimates. A significance level of 0.05 was established for all analyses.

3. Results

3.1. Epidemiological & clinical characteristics of the study population

The main epidemiological and clinical characteristics of the study population are presented in Table 2. The families of premature newborns had lower family income ($p = 0.023$), but the other socio-economic and demographic variables were similar between preterm and term groups. Regarding gestational health, except for the number of prenatal consultations, which was proportionally greater the higher the gestational age, the other variables were similar between the groups. Regarding birth-related and anthropometric variables, the significant differences found are explained by gestational age, and the distribution by sex had a similar proportion between the two groups.

3.2. Methylation percentage at different time points

The methylation levels are presented in Table 3, where the medians of methylation at each site (site-specific methylation) and the global methylation at the four sites studied (CpG 1 to 4) are observed. At birth (D0), significantly higher percentages of methylation were observed in the group of full-term newborns, both site-specific and global. On the fifth day of life (D5), the

Table 1. Target region used for primers design covering nucleotides 222 to 360, corresponding to the accession code X58075.1 in the NCBI GenBank. CpG sites of interest numbered and highlighted in bold.

Nucleotide No.	Nucleotide sequence
222	GTTCCTTT CCGAGTCAA GAAAGGGGTG ACGGACGCAC CTGGAAAATC
271	GGGTCACTCC CACCCGAATA TTGCGCTTTT CAGACC ¹ GGCT TAAGAAAC ² GG
321	C ³ GCACCA ⁴ GA GACTATATCC CACACCTGGC TCAGAGGGTC

Table 2. Epidemiological and clinical characteristics of pregnant women, preterm and term newborns.

Characteristics	Preterm (n = 46)	Full-term (n = 49)	p-value
Maternal age (years): median (IQR)	31 (25.2–34)	28 (24–34)	0.197 ^a
Maternal education (years): n (%)			0.474 ^b
1–4	1 (2.2)	0 (0)	
5–8	7 (15.2)	9 (18.4)	
9–11	8 (17.4)	13 (26.5)	
> 12	30 (65.2)	27 (55.1)	
Family income (BRL minimum wage): n (%)			0.023 ^b
< 1	13 (28.3)	4 (8.2)	
1–2	17 (37)	28 (57.1)	
> 2	16 (34.8)	15 (30.6)	
Ethnicity: n(%)			0.114 ^b
White	18 (39.1)	22 (44.9)	
Black	13 (28.3)	5 (10.2)	
Mixed race	15 (32.6)	22 (44.9)	
Health conditions			
Smoking: n (%)			> 0.999 ^c
No	44 (95.7)	46 (93.9)	
Yes	2 (4.3)	3 (6.1)	
Alcohol consumption: n (%)			0.323 ^c
No	42 (91.3)	48 (98)	
Yes	4 (8.7)	1 (2)	
Perinatal consultations: median (IQR)	5 (4–7)	9 (7–10)	< 0.001 ^a
Gestational age (weeks): median (IQR)	28.5 (27–30)	39 (38–40)	< 0.001 ^a
Apgar score (1 min): median (IQR)	7 (4.25–8)	8 (8–9)	< 0.001 ^a
Apgar score (5 min): median (IQR)	8.5 (8–9)	9 (9–9)	< 0.001 ^a
Sex: n (%)			0.634 ^b
Male	25 (54.3)	29 (59.2)	
Female	21 (45.7)	20 (40.8)	
Birth weight (g)			< 0.001 ^a
Mean (SD)	1074.5 (290)	3393 (385.0)	
Median (IQR)	1075 (860–1345)	3320 (3150–3575)	
Length (cm)			< 0.001 ^a
Mean (SD)	35.9 (1.8)	48.9 (3.4)	
Median (IQR)	36.4 (34–37.5)	49 (47.5–49.6)	
Head circumference (cm)			< 0.001 ^a
Mean (SD)	26 (2.4)	34.4 (1.5)	
Median (IQR)	26 (24.6–28)	34 (33.5–35)	

^aMann-Whitney U test.^bChi-square test.^cFisher's exact test.

BRL: Brazil; IQR: Interquartile range. SD: Standard deviation.

differences in the percentage of methylation between the groups decrease, remaining significantly higher in the full-term newborn group only at sites 2 and 4. The difference in global methylation did not reach statistical significance but resulted in a marginal *p*-value of 0.053. Figure 1 graphically illustrates the distribution of the global percentage of LINE-1 methylation in the full-term and preterm neonates group at birth (D0) and on the fifth day of life (D5), using violin plot box diagrams. Table 4 provides a detailed overview of LINE-1 methylation levels in full-term newborns (only D0 and D5) and in preterm neonates, stratified into the two

studied groups according to gestational age: extremely preterm (less than 28 weeks) and very preterm (28 to less than 32 weeks). Statistically significant differences observed on D0 and D5 between the full-term and very preterm groups are represented in Figure 2 and described more comprehensively in the following paragraphs. In Figure 2 (panels A and B), comparisons are shown between the percentage of global LINE-1 methylation at D0 and D5 among full-term and preterm infants, now stratified according to gestational age into very preterm and extremely preterm. In panel A, a significant difference in LINE-1 methylation is observed between full-

Table 3. Comparison of median methylation percentages between term and preterm infants at different time points.

		Full-term (%) (IQR)	Preterm (%)	<i>p</i> -value ^a
D0	CpG1	69.5 (68.2–70.7)	68.0 (66.45–70.4)	0.038
	CpG2	65.7 (64.4–67.3)	63.6 (64.47–67.3)	<0.001
	CpG3	69.7 (64.4–67.3)	68.3 (64.47–67.3)	0.012
	CpG4	78.0 (64.4–67.3)	75.2 (64.47–67.3)	<0.001
	CpG 1–4	70.9 (69.5–71.8)	68.1 (66.3–71.5)	0.002
D5	CpG1	70.4 (69.3–71.5)	69.8 (68.6–71.1)	0.112
	CpG2	66.5 (65.7–67.4)	65.9 (64.4–66.8)	0.020
	CpG3	70.4 (69.5–71.2)	70.4 (69.5–71.5)	0.931
	CpG4	79.5 (78.4–80.3)	78.6 (77.3–79.4)	0.010
	CpG 1–4	71.8 (71.1–72.4)	71.1 (70.0–72.1)	0.053
D30	CpG1	N/A	70.7 (70.0–71.7)	
	CpG2	N/A	66.0 (65.3–67.3)	
	CpG3	N/A	71.2 (70.4–72.1)	
	CpG4	N/A	79.5 (78.5–80.2)	
	CpG 1–4	N/A	71.6 (71.1–72.8)	
D90	CpG1	N/A	70.4 (69.8–71.1)	
	CpG2	N/A	66.4 (65.6–67.2)	
	CpG3	N/A	70.3 (69.6–71.5)	
	CpG4	N/A	78.3 (77.9–79.1)	
	CpG 1–4	N/A	71.3 (71.0–72.3)	

In bold: median percentage methylation for all CpGs (CpG 1–4) in full-term and preterm newborns.

^aMann-Whitney U test.

^bOne D0 sample could not be processed in the premature newborn group.

^cThe difference to the initial “n” represents samples lost to follow-up.

D0: Day of birth; D5: Fifth day of life; D30: Thirtieth day of life; D90: Ninetieth day of life; IQR: Interquartile range; N/A: Not available.

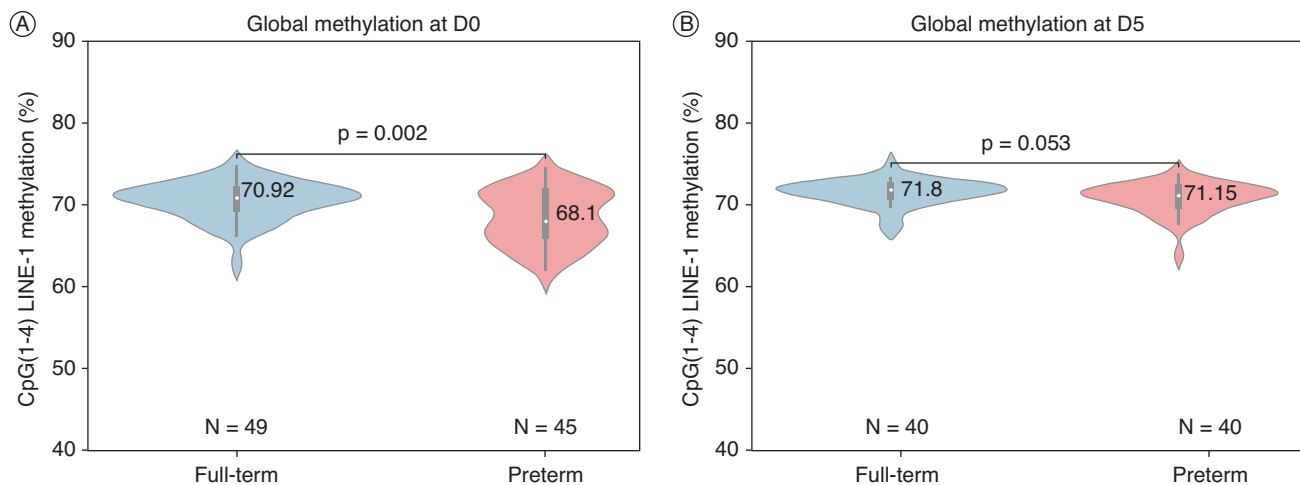


Figure 1. Distribution of global methylation percentage in full-term and preterm newborns. At birth (**A**), significantly higher methylation percentages were observed in the full-term newborn group. This percentage was still higher on the fifth day of life (**B**), but the difference between the groups did not show statistical significance, although with a marginal *p*-value.

Table 4. Comparison of global LINE-1 methylation percentage among full-term, very preterm^a and extremely preterm infants^b.

Time	n	Group	% of global methylation in LINE-1 (IQR)	Comparison with full-terms (<i>p</i> -value)	Comparison with very preterm (<i>p</i> -value)
D0	49	Full-term	70.92 (69.55–71.88)		
	31	Very preterm	67.21 (64.72–71.14)	<0.001	
	14	Extremely preterm	70.58 (67.70–71.52)	0.376	0.082
D5	40	Full-term	71.80 (71.05–72.40)		
	29	Very preterm	71.06 (69.32–71.77)	0.021	
	11	Extremely preterm	71.66 (71.06–72.28)	0.81	0.112
D30	25	Very preterm	71.54 (71.05–72.50)		
	11	Extremely preterm	72.50 (72.04–72.84)		0.164
D90	24	Very preterm	71.19 (70.64–71.53)		
	10	Extremely preterm	71.94 (71.21–72.55)		0.052

^aVery preterm: 28 to less than 32 weeks gestational age.

^bExtremely preterm: less than 28 weeks gestational age.

D0: Day of birth; D5: Fifth day of life; D30: Thirtieth day of life; D90: Ninetieth day of life; IQR: Interquartile range; n: Number of samples in each group.

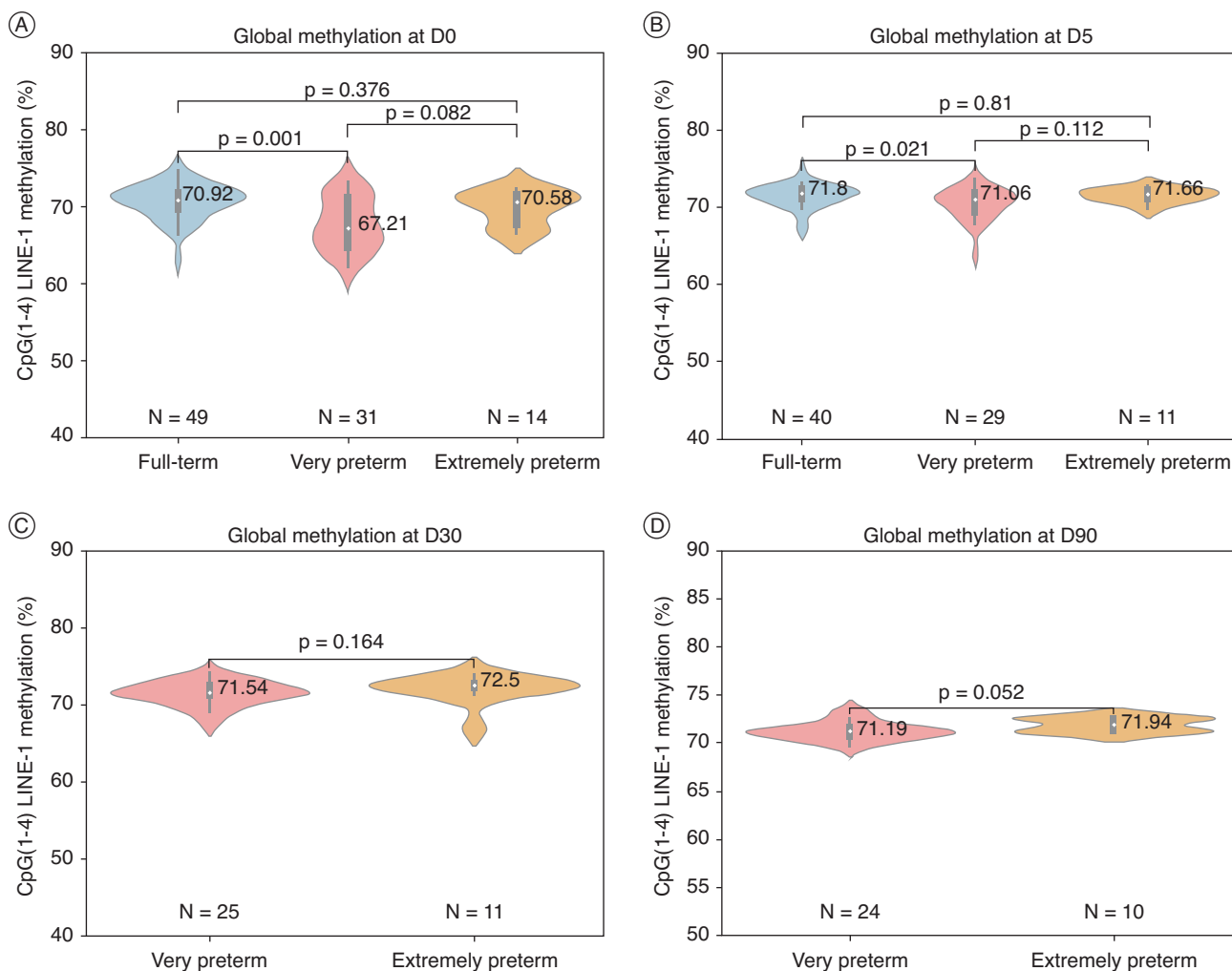


Figure 2. Distribution of global methylation percentage in term newborns, very preterm (28 to less than 32 weeks gestational age), and extremely preterm infants (less than 28 weeks gestational age) at birth **(A)**, on the fifth day of life **(B)**, on the thirtieth day of life **(C)** and the ninetieth day of life **(D)**. In panel A, a significant difference in methylation is observed between full-term and very preterm neonates, which is not observed between very preterm and extremely preterm. Extremely preterm infants had a methylation percentage closer to full-term newborns than to very preterm ones (not statistically significant). In panel B, the difference in global methylation between full-term and very preterm infants is still statistically significant, although not significant between very preterm and extremely preterm infants, as well as between full-term infants and extremely preterm infants. In panels **(C & D)**, there were no differences in the global methylation percentage between the subgroups of preterm infants, although the *p*-value on D90 **(D)** approached statistical significance.

term neonates (70.92%) and very preterm ones (67.21%), indicating a strong association between prematurity and a lower methylation percentage. However, comparing very preterm and extremely preterm infants showed no statistically significant difference. Extremely preterm infants had a median of 70.58% methylation, closer to full-term newborns than to very preterm ones, although this difference to full-term neonates was not statistically significant ($p = 0.376$). In panel B, it is observed that on D5, the difference in global methylation percentage between full-term infants and very preterm infants is still statistically significant ($p = 0.021$), a significance when full-term

infants were compared with the whole group of preterm infants ($p = 0.053$, Table 3 & Figure 1). However, the difference between very preterm infants and extremely preterm infants was not significant ($p = 0.112$), as well as between full-term infants and extremely preterm infants ($p = 0.81$). In panels C and D (Figure 2), comparisons are shown only between the strata of very preterm and extremely preterm infants, at D30 and D90, respectively. There were no statistically significant differences in the global methylation percentage between these groups ($p = 0.164$ on D30 and $p = 0.052$ on D90), although the *p*-value on D90 approached statistical significance.

3.3. Temporal evolution of DNA methylation in preterm neonates

The temporal evolution of methylation in preterm infants at the four specific CpG sites of the LINE-1 element (CpG1, CpG2, CpG3 and CpG4), from birth (D0) to the ninetieth day of life (D90), is shown in [Figure 3](#). Although a positive slope ($r = 0.50$ to 0.75) is observed at all four sites, this increasing trend was not statistically significant. Similarly, [Figure 4](#) displays the temporal evolution of global LINE-1 methylation at the four CpG sites from birth to 90 days of life. A positive slope is observed, but the upward trend is not statistically significant (stationary).

4. Discussion

This study revealed significantly reduced LINE-1 methylation levels in preterm neonates at birth compared with full-term newborns and that this pattern persists, at least at CpG sites 2 and 4, on the fifth day of life. We further demonstrated that this difference also occurs when comparing the overall methylation percentage between full-term newborns and the subgroup of very preterm neonates across the four studied sites. However, there appears to be a trend toward stabilization of LINE-1 methylation levels in the group of preterm newborns over the first 90 days of life, reaching values similar to those found in full-term newborns at birth, since the fifth day onwards.

Although the aim of this study was not to describe the risk factors for prematurity, these factors may be associated with epigenetic alterations, including those of the LINE-1 element. We found lower family income in the preterm group, which is related to various conditions that can negatively influence LINE-1 methylation, such as exposure to air pollution, metals poisoning, inadequate diet, obesity, chronic stress and smoking [37–40]. Conversely, a diet rich in vegetables and isoflavones, zinc supplementation and physical exercise can positively influence this methylation [30,41–43]. Folic acid, especially when supplemented during pregnancy, not only prevents the invasive behavior of the trophoblast and the risk of pre-eclampsia but also has a positive effect on LINE-1 methylation [44].

Essentially, this study suggests a direct influence of prematurity on the initial epigenetic configuration by revealing variations in the methylation of specific CpG sites of LINE-1, particularly lower in preterm infants at birth. However, the observed differences seem to attenuate in the first days of life, indicating a process of normalization in the epigenetic profile of preterm infants after birth, which may have implications for understanding neonatal development in this group of patients. Furthermore, although there is a positive inclination in

the percentage of LINE-1 methylation over time, this progression has not proven to be a statistically significant trend (stationary), which stabilized at proportions comparable to the values found in the full-term neonate group from birth to the fifth day of life. This pattern suggests ongoing adaptive or recovery processes and a possible progressive refinement of the epigenome (maturation) in the first months of life, regardless of prematurity. The higher the global levels of methylation (LINE-1), the greater the capacity to maintain the structure and sequence of the genetic material over time without undesirable changes or significant mutations (genomic integrity) [45]. Therefore, the dynamic nature of this methylation could reflect postnatal adaptations and development for both term and preterm newborns, with possible implications for both genomic stability and gene expression. The analysis of these data opens a field for discussion, regarding its biological implications and the intersections between birth conditions, socioeconomic context and neonatal health.

An intriguing finding of our study was the high percentage of methylation in the subgroup of extremely premature newborns. Considering the greater prematurity, the expected result would be the opposite: a lower proportion of methylation in this group and a greater difference, especially concerning full-term newborns, but also regarding premature neonates of greater gestational age. However, what was observed was a percentage of methylation close to that of full-term newborns and higher than that observed in the group of very premature infants. Recent studies have highlighted that LINE-1 activation after fertilization is crucial for global chromatin accessibility in the early embryo, particularly at the 8-cell stage. Inadequate LINE-1 activation can result in preimplantation development defects and spontaneous abortion [16,19,46]. Thus, a high percentage of methylation in the LINE-1 promoter (indicating lower activity) may have contributed to extremely premature birth. In addition, some stressful factors associated with extremely premature birth may have influenced the LINE-1 methylation status, including epigenetic interactions with other genes, or simply the small number of patients in this subgroup may have affected the statistical results. In any case, future studies will be able to investigate this aspect better.

Prematurity's complexity and impact on neonatal development are vital topics in neonatal and pediatric research. A recent study conducted by Fontana et al. (2021) sheds light on the intersection between prematurity, DNA methylation in the LINE-1 element, and the neurocognitive development of neonates [47]. The authors found that the LINE-1 promoter was hypomethylated in preterm neonates at birth, but this methylation

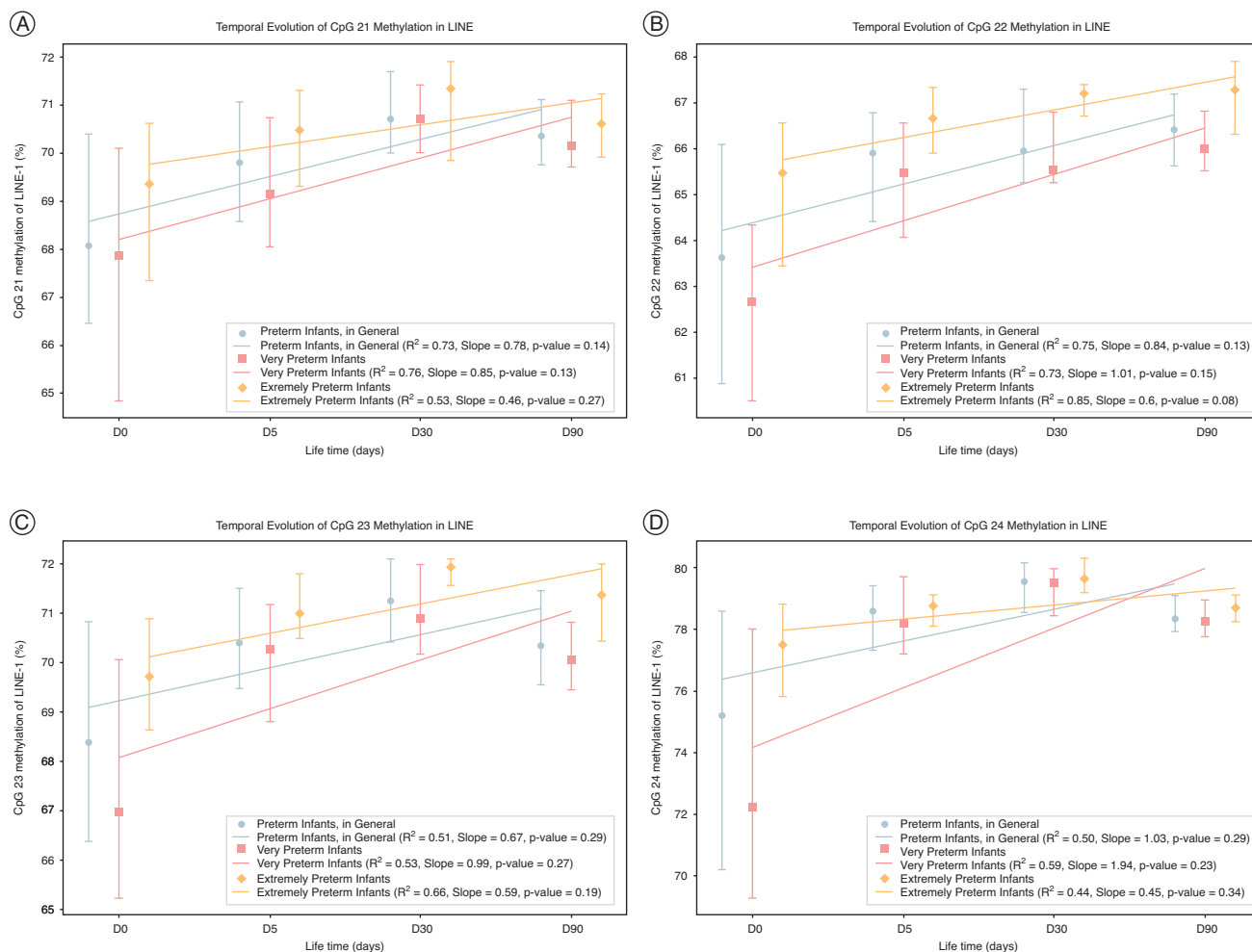


Figure 3. Temporal evolution of site-specific methylation in preterm newborns at CpG sites 1 (A), CpG 2 (B), CpG 3 (C) and CpG 4 (D) of the transposable element LINE-1 at time points D0, D5, D30 and D90, showing positive slopes but not significant trends.

was restored due to early special maternal care, which may demonstrate the neonatal epigenome's sensitivity to environmental factors, especially in premature infants. Furthermore, the group randomized to enhanced maternal care showed higher developmental scores at 12 and 36 months of life than the standard care group. This suggests not only that early special maternal care may restore LINE-1 methylation levels in preterm infants but also that this is associated with better outcomes in children's neurocognitive development. Although we studied different subtypes of LINE-1 and the premature infant group received only standard care, we observed restoration of LINE-1 methylation (values similar to those of full-term newborns at birth) as early as the fifth day of life, which remained stable (stationary trend) until 90 days. Not disregarding the possible benefit of special care for the newborn and its positive influence on neurodevelopment, our findings make it possible to wonder whether the restoration of the LINE-1 methylation pattern

would occur as a natural adaptation process in premature newborns.

In our opinion, some points can be highlighted in our findings: methylation was investigated at several time intervals (D0, D5, D30 and D90), which allows a more comprehensive view of the evolution of LINE-1 methylation status in the neonatal period and first months of life in premature infants; the sample size is larger than previously studied; and the pyrosequencing technique used (Pyromark Q48 Autoprep) is more modern than the Sanger method, providing greater precision and sensitivity. Unfortunately, due to differences in the techniques used and the CpGs analyzed (originating from different consortia: X58075.1 and L19092.1), the methylation values identified by our study cannot be directly compared with those in the study of Fontana et al. However, both studies converge to highlight prematurity as a significant factor that affects (reduces) DNA methylation in the LINE-1 element, which may have

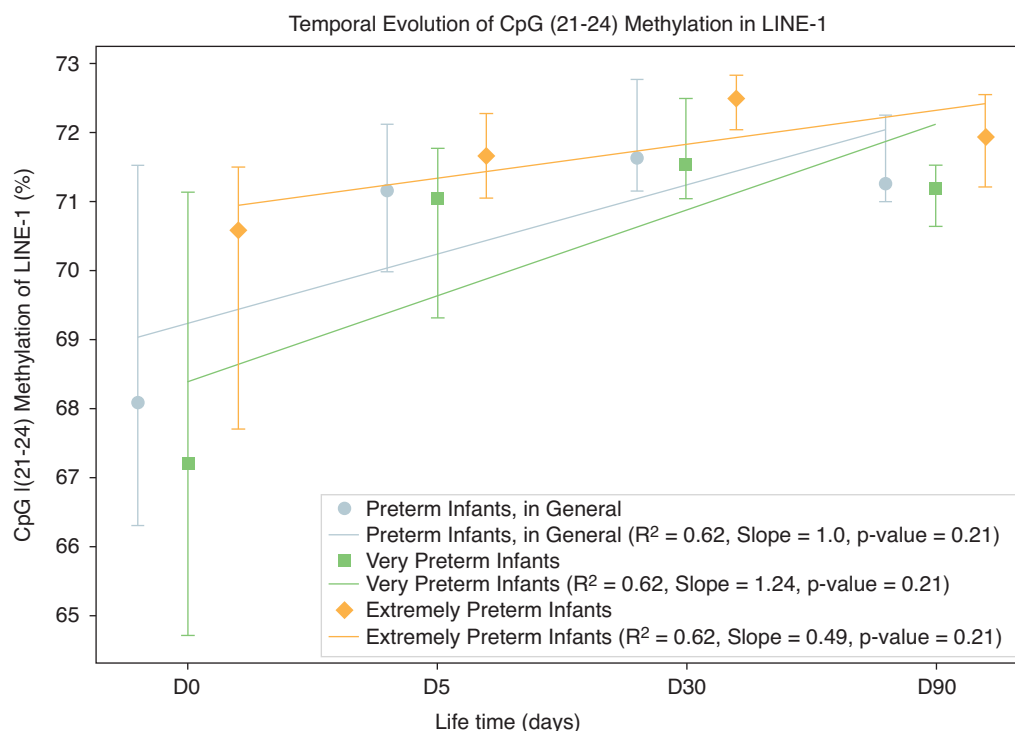


Figure 4. Temporal evolution of global methylation in LINE-1 CpG sites in preterm newborns at D0, D5, D30 and D90 shows a positive slope but not a significant trend.

implications for the neurocognitive development of these patients.

The relationship between LINE-1 methylation and neurological and psychiatric outcomes is an emerging topic in neuroscience and psychiatric research. The findings of this research regarding low global methylation (LINE-1) in preterm neonates, at least at birth and in the early days of life, gain greater significance when considering recent studies that associate alterations in LINE-1 methylation with psychiatric diseases, including autism spectrum disorder (ASD), post-traumatic stress disorder and panic disorders [48,49]. These associations underscore the importance of studying the methylation state of LINE-1 as a potential biomarker and influencing factor in the development of psychiatric conditions, especially in the preterm newborn population. Furthermore, some studies suggest that methylation status of repetitive elements like Alu and LINE-1 in humans is considered a surrogate for global DNA methylation [20]. Applying these findings to the context of child neurodevelopment raises concerns that low LINE-1 methylation in preterm newborns, by implicating global methylation, may also affect the expression of genes essential for neurodevelopment.

This study presents some strengths and limitations. The main strength lies in the originality of investigating

global methylation (LINE-1) using an advanced genetic sequencing technique in human samples of term and, notably, preterm newborns. Among the limitations, we highlight the convenience sample and the small sample size, determined by logistical and budgetary issues. Assessing the exact statistical power became unfeasible due to the lack of preliminary studies that could support an accurate calculation of the sample size; the absence of data on the percentage of methylation for full-term newborns at D30 and D90, as these samples were fully used in a previous study conducted with the same cohort [33], which made comparative analysis at these time points impossible; the lack of evaluation of potential transgenerational inheritance in LINE-1 methylation; and LINE-1 methylation serves only as a marker of global DNA methylation. Consequently, establishing its relationship with specific gene expression is challenging. Thus, further research in this area may show that changes in LINE-1 methylation can reflect broader shifts in the epigenetic landscape, which may influence gene expression, neurological development and other areas of human health. Therefore, studying LINE-1 methylation in preterm neonates and following these cohorts can provide valuable insights into brain development risks, mental health and overall health later in life.

5. Conclusion

This study demonstrated that preterm newborns exhibit lower methylation percentages in the promoter region of the transposable element LINE-1 compared with full-term newborns at birth and in the first days of life, reflecting the influence of prematurity on the initial epigenetic configuration. However, there appears to be a trend toward stabilizing this percentage over the first 90 days of life in the preterm group, suggesting a possible dynamic process of epigenetic maturation that could be related to adaptations to the postnatal environment. These findings underline the complexity of epigenetic regulation in response to prematurity and emphasize the need for further studies that can link the epigenome to child development.

Article highlights

- Premature infants exhibit significantly lower levels of LINE-1 promoter methylation at birth compared with full-term infants.
- The subgroup of extremely premature newborns showed higher percentages of LINE-1 methylation than expected, greater than the very premature subgroup and not significantly different from full-term newborns.
- These epigenetic differences suggest that prematurity affects the initial epigenetic configuration of newborns.
- The study analyzed blood samples from both premature and full-term newborns collected at birth and at various time points in early life (D5, D30 and D90).
- DNA methylation patterns were assessed using sodium bisulfite conversion and pyrosequencing techniques.
- The study contributes to the growing body of knowledge on the epigenetic mechanisms underlying neonatal health challenges.

Acknowledgments

The authors would like to thank the staff at the Obstetric Center and the Neonatal ICU at the UFRJ Maternity School for their assistance in collecting data for this research.

Author contributions

A Prata-Barbosa, AJL Alves da Cunha, MC de Magalhães-Barbosa and PVB Eleutério dos Santos conceived and designed the study. G Chalfun, M Santos Salú, MB Genuíno de Oliveira and JR Robaina performed data collection and management. PVB Eleutério dos Santos, A Araújo Brasil, LT Vieira Milone, SS and M Santos Salú performed the laboratory part of the study. G Rodrigues-Santos, F Lima-Setta and LT Vieira Milone performed the statistical analysis. PVB Eleutério dos Santos, AAB and APB drafted the manuscript, and all authors revised the manuscript critically and approved the final version.

Financial disclosure

Funding for the presented work was provided by the Department of Pediatrics of D'Or Institute for Research and Education (IDOR), Rio de Janeiro, RJ, Brazil, www.idor.org. Carlos Chagas Filho Foundation for Research Support in the State of Rio de Janeiro (FAPERJ), grant n° E-26/210.061/2024.

Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate approval from the Research Ethics Committees of the Maternity School of the Federal University of Rio de Janeiro (No. 2,529,806) and the D'Or Institute for Research and Education (No. 2,432,638) and have followed the principles outlined in the Declaration of Helsinki. In addition, written informed consent has been obtained from the participant's parents or legal guardians.

Data availability statement

Data from this study can be accessed at the following link: <https://github.com/PauloBarbosa2002/Impact-of-Prematurity-on-LINE-1-Promoter-Methylation.git>

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